FUNCTIONAL CHARACTERIZATION OF RFX6 AND SEL1L IN PANCREATIC DEVELOPMENT

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ABSTRACT

During vertebrate embryonic development, a common pool of progenitor cells gives rise to all three lineages of the pancreas, including endocrine, exocrine and duct cells. The molecular mechanisms regulating pancreatic differentiation are incompletely understood. We investigated the function of two genes in the control of pancreatic differentiation. In chapter two, we show that *Sel1L* (Sel-1 suppressor of lin-12-like) is a potential Notch regulator during pancreas development. SEL1L is initially expressed throughout the pancreatic epithelia and later restricted in differentiated cells. Mice lacking SEL1L show severe defects in the differentiation of both endocrine and exocrine pancreas. These differentiation defects can be rescued by the Notch signaling inhibitor, DAPT. In chapter three, we show that RFX6 (Regulatory factor X 6) functions as a transcription factor in the developing pancreas. Knockdown of RFX6 in zebrafish causes disorganized pancreatic morphology and attenuated insulin expression. Expression of *Nkx6.1* is down regulated in pancreatic cell line transfected with *Rfx6* SiRNA. Luciferase reporter studies reveal that RFX6 activates the proximal promoter of *Nkx6.1*. In summary, our studies have provided new insight in the spatial and temporal regulation underlying pancreas development.

BIOGRAPHICAL SKETCH

Shuai Li was born on December 11th, 1984, in Chengdu, a city in the southeast of China full of culture and tradition where he spent his childhood. At the age of 11, he was sent to the full time boarding school, Chengdu Experimental Foreign Language School where he received intensive trainings in English. Immediately after graduation from high school, he was admitted to Peking University for undergraduate education and later received his Bachelor of Science in Biological Science in 2002. Shuai came to the United States for graduate studies in 2007 and he was admitted to the Department of Animal Science at Cornell University. During this period, he worked under the instruction of Dr. Qiaoming Long on the projects related to diabetes research. After completing his Master of Science degree in July of 2010, Shuai will continue his study for the Master of Science in Bioengineering at University of Pennsylvania.

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LIST OF ABBREVIATIONS

SEL1L: Suppressor enhancer of lin-12 like

RFX6: Regulatory factor X 6

PTF1A: Pancreas Transcription Factor 1, Alpha Subunit

NGN3: Neurogenin 3

IA-1: Insulinoma-associated protein 1

FGF: Fibroblast growth factor

BMP: Bone morphogenetic protein

TGF-β: Transforming growth factor beta

CHAPTER 1

INTRODUCTION

Diabetes is metabolic disease characterized by hyperglycemia and chronic organ damage. The substantial increase of diabetes has become one of the greatest concerns in human health. In 2008, over 24 million people in the United States were diagnosed with diabetes, along with 57 million people estimated to have pre-diabetes [1]. Diabetes consists of 2 major types, type 1 and type 2. In type 1, β cells are destroyed by autoimmune disorders [2]. In type 2 Diabetes, hyperglycemia is caused by insulin resistance in the peripheral organs and the inability of the pancreas to compensate via insulin secretion [3].

Currently daily insulin injection is the only treatment for type 1 diabetes and for some cases of severe type 2 diabetes [4, 5]. Islets transplantation has been proven to be a promising treatment for type 1 diabetes [6, 7]. However, clinical application of islets transplantation is limited by autoimmune response after transplantation and the source of islets donors. Potential alternative sources of β cells include differentiated embryonic and adult stem cells. Thus a complete understanding of the molecular factors regulating β cell formation will greatly facilitate the development of cell-based therapies [9, 10, 11].

1.1 Morphogenesis during pancreas development

The pancreas is a bi-functional organ composed of 3 different cells types, exocrine cells that excrete digestive enzymes, endocrine cells that secrete peptide hormones and the multi-branched pancreatic ducts that channels digestive enzymes. The endocrine compartment is organized as the islets of Langerhans, with each islet

consisting of five subtypes of cells, α cells that secrete glucagon, β cells that secrete insulin, δ cells that secrete somatostatin, PP cells that secrete pancreatic polypeptide and ε cells that secrete ghrelin [12, 13, 14].

Pancreas development in mice starts at approximately e9.0 as a thickening patch of epithelial cells from the primitive foregut endoderm. These cells co-express *Pdx1* and *Ptf1a*, the earliest and most specific genes that determine a pancreatic fate [15, 16]. Later, a second *Pdx1/Ptf1a* co-expressing domain is defined on the ventral side of the primitive foregut [20, 21, 22]. The two pancreatic epithelial domains thicken and form an observable dorsal bud at e9.5 and ventral bud at e10.5. Between e10.5 to e12.5, the two buds branch out to form a glandular structure with several folds increase in size. In the meantime, rotation of the gut brings the ventral bud to the dorsal side and the two buds fuse into one interconnected pancreatic bud on e12.5 [23, 24].

The endocrine and exocrine cells of the pancreas differentiate asychronically during pancreatic development. Scattered endocrine cells appear as early as e9.5. These cells express either glucagon or insulin [25, 26]. This event of early endocrine cell differentiation is known as the primary transition. Following the primary transition, a great number of endocrine cells start to appear in the center of the developing pancreas at e13.5, along with amylase expressing cells emerging on the peripheral region. This synchronically differentiation of endocrine and exocrine cells is known as the secondary transition [27, 28]. It has been suggested that the primary and secondary transition are regulated by distinct transcriptional programs [25, 29]. Genetic disruption of the essential transcription factor *Pdx1* eliminates both pancreas development and endocrine cells from the secondary transition, but the endocrine cells from the primary transition are not affected [30, 31].

Both endocrine and exocrine compartments grow significantly during neonatal period in order to meet the physiological demand after birth and weaning [32]. During this period, endocrine cells migrate to form islets that scatter throughout exocrine tissues. The adult pancreas can expand and regenerate its lost tissue in response to pathological and physiological conditions [33, 34].

1.2 **Signaling pathways controlling pancreatic development**

The Hedgehog pathway signaling controls the initiation and positioning of the pancreatic endoderm at early development stages [37]. Hedgehog inhibits early *Pdx1* expression and hence suppresses pancreas formation. During early embryonic development, hedgehog is expressed throughout the primitive foregut endoderm but inhibited on the pancreatic region, permitting the expression of *Pdx1*. This critical inhibition of hedgehog results from the surrounding environment of the primitive foregut endoderm. Previous studies have shown that specification of the dorsal pancreatic endoderm is induced by activin signaling from the adjacent notochord [17, 18, 19]. In the ventral bud, down-regulation of hedgehog is correlated with epithelium migration from its originally high levels of FGF and BMP environment, which normally stimulate hedgehog signaling [38].

Endothelial signals from the dorsal aortas are essential for maintaining the proliferation of PDX1-positive pancreatic endoderm at early stages. It has been found that the endothelial signals also induce *Ptf1a*, another crucial transcription factor that contributes to the determination of pancreatic endoderm. Endothelial signaling also contributes to the vascularization in the developing and mature pancreas [39, 40, 41].

Signals from the surrounding mesenchymal tissue play an important role in the morphogenesis and cellular differentiation of the embryonic pancreas. Epithelium

separated from its surrounding mesenchyme fails to expand and to differentiate *in vitro*. However, the normal development of the pancreatic epithelium is restored when co-cultured with mesenchyme [42]. These evidences suggest that the surrounding mesenchyme tissue provides essential signals for pancreas development and differentiation.

Recent studies have revealed that Wnt signaling promotes proliferation while suppresses differentiation of the PTF1A-positive cells. In contrast, Wnt signaling has little effect on the proliferation and differentiation of the endocrine progenitor cells [43].

FGF family members direct the growth and differentiation of the pancreas. Multiple FGF and FGF receptors have been identified in the developing pancreatic mesenchyme and epithelium, including FGF1, FGF2, FGF7, FGF9, FGF10 and FGF11 [44]. FGF2 promotes the expansion of dorsal pancreatic endoderm [37] while suppresses the expansion of ventral pancreatic endoderm [45]. FGF10 is secreted by the pancreatic mesenchyme starting from e10.5. Genetic disruption studies of FGF10 in mice have indicated its role in both inducing PDX1-positive epithelial cells and later promoting their rapid proliferation [46, 47]. FGF10 also maintains the undifferentiated states of pancreatic progenitor cells. It has been suggested that FGF10 prevents differentiation through cross-talking with Notch signaling and up-regulating the expression of its effector gene *Hes1* [48, 49].

Notch signaling is shown to play a critical role in balancing proliferation and differentiation of the multipotent progenitor cells pool. Its detailed function and regulation are described in section 1.4.

1.3 Transcriptional regulation of pancreas differentiation

Pdx1 expression starts at e8.5 in the primitive foregut endoderm. The expression of *Pdx1* later becomes restricted in the central region of the epithelium, which later adopts an endocrine fate. In the adult pancreas, *Pdx1* is exclusively expressed in the islets [31]. Previous studies on insulin transcriptional regulation have revealed that PDX1 directly binds to the insulin proximal promoter and activates its transcription [54].

Genetic disruption of *Pdx1* causes pancreas agenesis, indicating an essential role in pancreas development. However, the presence of a small number of endocrine cells in intestine of the PDX1-null mice suggests that an alternative endocrine lineage exists independent from Pdx1 induction [55].

Ptf1a, or *p48*, is a subunit in the heterooligomeric transcription complex PTF1 that regulates the expression of a large number of genes in the exocrine pancreas. *Ptf1a* expression is confined in a subset of the *Pdx1* expressing cells at e9.5. *Ptf1a* expression becomes progressively exclusive in the periphery of the branching pancreatic epithelium that commits to exocrine fate. Knockout of *Ptf1a* causes major defects in pancreas development and only pancreatic remnants can be observed after birth [56].

Both *Pdx1* and *Ptf1a* play fundamental roles in pancreas development. It has been generally accepted that the pancreatic endoderm is defined by the overlapping expression domains of the two transcription factors. Double knockout of *Pdx1* and *Ptf1a* have revealed that *Pdx1* is required in a stage prior to *Ptf1a* in specification of pancreatic epithelia. In addition, *Pdx1* is required for sustaining the transient expression of *Ngn3* from e9.5 and thus is essential for defining the endocrine lineage in the second transition. In contrast, *Ptf1a* displays only limited influences on the differentiation of endocrine cells [57].

Ngn3 functions as a master switch for determining an endocrine cell fate. Genetic ablation of NGN3 completely devoids all endocrine cell types. During development, expression of *Ngn3* is transiently detected in the pancreatic epithelia from e9.5. Its expression progressively increases and peaks at e15.5 within the cells expressing higher level of *Pdx1*. Later, *Ngn3* expression declines rapidly and becomes undetectable after birth. During its transient expression, *Ngn3* activates a series of downstream transcription factors that are required for the differentiation of endocrine cells [58, 59].

NeuroD1 was identified as a direct target for *Ngn3*. The expression of *NeuroD1* starts in all pancreatic endocrine cells including endocrine precursors from e9.5. *NeuroD1* knock-out mice display severe defects in pancreatic development characterized by disorganized islets [60]. *IA-1* is another target activated by the transcription factor *Ngn3*. Genetic disruption of *IA-1* causes severe impairment of β cells differentiation. IA-1-null pancreas accumulates of endocrine precursor cells without hormone expression, suggesting that IA-1 might play a role in endocrine cell maturation [61].

Several transcription factors regulate beta cell differentiation and maturation. *Nkx6.1* has been indentified as a transcription repressor that promotes β cell differentiation. Gene targeting of *Nkx6.1* in mice causes significant reduction of islets. *In vitro* studies have shown that *Nkx6.1* is essential for promoting β cell differentiation and survival after the secondary transition [62]. *MafA* directly activates insulin promoter similar to *Pdx1*. Its expression starts in the primitive insulin-positive cells and continues in the mature islets. *MafA* knockout animals have normal pancreas with no significantly reduced β cell number or insulin expression. However, newborns rapidly develop impaired glucose tolerance with defects in insulin secretion, suggesting its role in beta cell maturation [63].

RFX3 is a member of the regulatory factor X family. Analysis of *Rfx3* knockout mice reveals *Rfx3* is required for β cell maturation. Furthermore, *Rfx3* has been shown to directly activate the transcription of glucokinase, a β cell specific glucose sensory protein involved in insulin secretion [64, 65].

1.4 The Regulation of Pancreatic Progenitor Cells Differentiation by Notch Signaling

Notch signaling has been shown to play a critical role in regulating proliferation and differentiation of the multipotent progenitor cells pool. Multiple Notch family members, including Notch 1-4 and the membrane bound ligands, DLL1, DLL3, Jagged 1, Jagged 3, Serrate 1 and Serrate 2 are expressed in the developing pancreatic epithelium and mesenchyme [19]. The Notch effector gene, *Hes1*, inhibits the transcription of *Ngn3*, a crucial transcription factor determining endocrine cell fate [50]. During early pancreas development, activated Notch signaling promotes proliferation of pancreatic progenitor cells by suppressing both endocrine and exocrine cell differentiation. Down-regulation of Notch signaling permits exocrine and endocrine cell fate commitment. Transgenic overexpression of notch intracellular domain (NICD) attunes both endocrine and exocrine cell differentiation [51, 52]. In contrast, genetic inhibition of notch causes premature pancreas [53].

During pancreas development, the interaction between the membrane bound ligand Delta or Serrate and the receptor notch determines binary cell fate through a process known as lateral inhibition [66, 67]. In pancreatic progenitor cells, activated notch receptor is cleaved and its intracellular domain, NICD, is released. NICD translocates into the nucleus and activates the Notch effector gene *Hes1* [68]. As a

transcription repressor, HES1 prevents endocrine cell differentiation by inhibiting the transcription of *Ngn3*. In addition, HES1 directly inhibits *p57*, a cyclin dependant kinase inhibitor that sustains cell cycle arrest [69, 70]. Notch signaling thus inhibits differentiation and maintains proliferation of the progenitor cells pool. Notch signaling is also modulated by other factors through their regulation of NICD and *Hes1* [71, 72, 73].

Recently, other transcription factors, including *Onecut 1, Foxa2, Tcf2* and *Sox9*, have also been indentified to be important for sustaining progenitor cells pool [74, 75, 76, 77]. *Sox9*, a transcription factor highly expressed in the pancreatic multi-potent progenitor cells is shown to be the transcription factor that governs this network by coordinately activating *Onecut 1, Foxa2* and *Tcf2*. In addition, Sox9 potentially regulates the expression of *Hes1* during the secondary transition [78, 79, 80].

1.5 Objectives

In chapter two, the objective was to investigate the regulation of Notch signaling in the developing pancreas. *Sel1L*, suppressor enhancer of lin-12 like, is a mammalian homolog of Sel-1 in *C.elegans. Sel1L* was first identified as a Notch repressor in *C.elegans* [81]. In mice, *Sel1L* is first expressed at e9.5 in the specified pancreatic buds [82, 83]. It has been hypothesized that *Sel1L* acts as a Notch repressor during pancreatic development. We studied the function of *Sel1L* using a gene-trap mice model where *Sel1L* was truncated in the fourteenth intron by the β-geo cassette. In the heterozygous mice line, the *Sel1L* expressing cell lineage can thus be traced by the expression of β-geo. Studies on the pancreas development of the *Sel1L* homozygous mutant were performed to unravel its function during endocrine and exocrine cell differentiation. Finally, Notch inhibitor DAPT was used in the mutant

explants culture to confirm the relationship between Notch activity and *Sel1L* deficiency.

In chapter three, the objective was to testify the function of RFX6 in controlling β cell differentiation. RFX6 belongs to the Regulatory Factor X family that attracted great attention in recent pancreatic development studies [84]. Our previous gene differential expression studies have revealed its exclusive expression in the developing pancreatic epithelium and in the adult islets. These evidences led to the hypothesis that *Rfx6* is a novel transcription factor involved in controlling differentiation of β cells. We first used zebrafish as a genetic model to study the function of RFX6 in pancreas development. In addition, we attempt to identify the possible transcription factors that are regulated by *Rfx6* in order to place it in the hierarchy of directing β cell differentiation.

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CHAPTER 2

SEL1L DEFICIENCY IMPAIRS GROWTH AND DIFFERENTIATION OF PANCREATIC EPITHELIAL CELLS*

2.1 Abstract

Background: The vertebrate pancreas contains islet, acinar and ductal cells. These cells derive from a transient pool of multipotent pancreatic progenitors during embryonic development. Insight into the genetic determinants regulating pancreatic organogenesis will help the development of cell-based therapies for the treatment of diabetes mellitus. Suppressor enhancer lin12/Notch 1 like (Sel1l) encodes a cytoplasmic protein that is highly expressed in the developing mouse pancreas. However, the morphological and molecular events regulated by Sel1l remain elusive.

Results: We have characterized the pancreatic phenotype of mice carrying a gene trap mutation in Sel1l. We show that Sel1l expression in the developing pancreas coincides with differentiation of the endocrine and exocrine lineages. Mice homozygous for the gene trap mutation die prenatally and display an impaired pancreatic epithelial morphology and cell differentiation. The pancreatic epithelial cells of Sel1l mutant embryos are confined to the progenitor cell state throughout the secondary transition. Pharmacological inhibition of Notch signaling partially rescues the pancreatic phenotype of Sel1l mutant embryos.

Conclusions: Together, these data suggest that Sel1l is essential for the growth and differentiation of endoderm-derived pancreatic epithelial cells during mouse embryonic development.

* Li S, Francisco AB, Munroe RJ, Schimenti JC, Long Q. SEL1L deficiency impairs growth and differentiation of pancreatic epithelial cells. BMC Dev Biol. 10:19 2010

2.2 Background

The multiple cell types that make up the adult pancreas, including endocrine, exocrine and ductal cells, derive from a common pool of pancreatic progenitors. Pancreatic development in mice begins at embryonic day 9.5 (E9.5) with the formation of two epithelial buds on the dorsal and ventral side of the primitive gut endoderm [1]. Epithelial cells within the pancreatic buds proliferate rapidly and branch out during later embryonic days to form a complex tubular network comprised of undifferentiated multipotent progenitor cells [2, 3]. Starting at E13.5, the expanded pancreatic epithelial cells undergo an asynchronized wave of differentiation to give rise to all the differentiated cell types of the adult pancreas, including acinar cells that produce hydrolytic digestive enzymes and islet cells that secrete endocrine hormones [4, 5]. Pancreatic morphogenesis depends on a complex and yet incompletely characterized network of transcription factors. Significant efforts have been made in the past few years to understand the role of several important transcription factors, including *Pdx1* [6, 7], *Ptf1a* [8, 9], *Sox9* [10, 11], *Ngn3* [12, 13], *NeuroD1* [14, 15], *Pax4* [16], *Pax6* [17], *Nkx2.2* [18], *Nkx6.1* [19], *Arx* [20], *Isl1* [21] and *Insm1* [22]. It is generally accepted that these transcription factors coordinate pancreatic morphogenesis by functioning in concert to restrict the developmental potentials of the pancreatic progenitors in a spatial and stage-specific manner [23].

Several previous studies have underscored the importance of Notch-mediated signaling in regulating pancreatic cell proliferation and cell fate decisions through control of *Ngn3* gene expression. During pancreatic development, *Ngn3* is transiently expressed in a subset of the pancreatic epithelial cells. NGN3 deficiency completely abolishes formation of all the endocrine cell subtypes, suggesting *Ngn3* functions as a master switch for the endocrine lineage in the pancreas. Mutations in genes encoding Notch signaling pathway components, such as DLL1 (ligand), RBP-Jk (the

intracellular mediator), or HES-1 (the effector) causes expansion of Ngn3 expression in pancreatic cells and, as a result, accelerated differentiation of endocrine cells at the expense of acinar and ductal cells [13, 24, 25]. Conversely, over or persistent expression of the Notch intracellular domain (NICD), a constitutively active form of Notch receptors, or the Notch effector gene *Hes1* results in diminished expression of *Ngn3* and attenuated differentiation of endocrine cells [26-28]. These studies suggest that during pancreatic development Notch signaling controls the endocrine and exocrine cell fate decisions of pancreatic epithelial cells by directly regulating *Ngn3* expression. Recent studies have also indicated the importance of Notch signaling in control of exocrine cell differentiation. Ectopic expression of activated NOTCH-1 prevents or significantly delays differentiation of acinar cells [26, 27].

While the role of Notch signaling in control of pancreatic cell proliferation and cell fate decisions is clearly recognized, the molecular mechanisms necessary for proper control of Notch signaling during vertebrate pancreatic development are poorly understood. Genetic and biochemical studies in invertebrates suggest that regulation of Notch signaling occurs at various levels and through multiple mechanisms [29, 30, 31]. These include stochastic and/or developmental expression of the Notch receptors and their ligands [32, 33, 34], selective receptor-ligand interactions [35, 36], intracellular protein trafficking [37] and stability of NICD [38]. *Suppressor enhancer lin12 1 like (Sel1l*) encodes a cytoplasmic protein that is highly conserved throughout the vertebrate kingdom [39]. RNA *in situ* hybridization and immunohistological analysis revealed that *Sel1l* is highly expressed in both the embryonic and adult pancreas [40, 41, 42]. The human *Sel1l* gene is located in a chromosome region that is in close proximity to a type 1 diabetes high risk locus, IDDM-11 (insulin-dependent diabetes mellitus locus 11), prompting the speculation that mutations in *Sel1l* may be associated with the pathogenesis of type 1 diabetes [43]. *Sel-1*, the *C. elegans* ortholog

of *Sel1l*, was first identified in a genetic screen for mutations that suppress lin-12/Notch activity [44]. Subsequent biochemical studies demonstrated that *Sel-1* negatively regulates lin-12/Notch activity by controlling lin-12/Notch turn-over [45, 46]. Based on these findings, it has been suggested that *Sel1l* may also function as a negative regulator for Notch signaling [47]. Recent biochemical and molecular studies *in vitro* revealed that *Sel1l* is also required for maintaining homeostasis of the endoplasmic reticulum (ER). SEL1L nucleates an ER membrane protein complex that is required for dislocation of unfolded or misfolded proteins from the ER lumen into the cytosol for degradation [48, 49].

In an attempt to decipher the developmental and physiological roles of *Sel1l*, we have generated and characterized mice carrying a gene trap mutation in the *Sel1l* gene. We report here that *Sel1l* expression during pancreas development coincides with differentiation of pancreatic epithelial cells into both the endocrine and exocrine lineages. Homozygous *Sel1l* mutant embryos exhibit an impaired pancreatic epithelial growth and branching morphology. Pharmacological inhibition of Notch signaling rescues the pancreatic phenotype of *Sel1l*-deficient embryos. These data are consistent with the notion that *Sel1l* functions as a negative regulator for Notch signaling during pancreatic organogenesis [47].

2.3 Results

Sel1l expression during mouse pancreatic development

We generated mice carrying a gene-trap insertion in the *Sel1l* gene. The gene trap cassette, located in intron 14, contains a β-galactosidase-neomycin (βgeo) fusion reporter gene. To determine the spatiotemporal expression pattern of *Sel1l* in the developing mouse pancreas, we performed immunohistological analysis of pancreatic sections from $Sel1l^{+/\beta geo}$ embryos using antibodies against β-galactosidase (βgal) and

several pancreatic proteins, including SOX9 and PDX1 (progenitor markers), insulin and glucagon (endocrine lineage markers) and amylase (exocrine lineage marker). βgal expression was first detected at E12.5 in a small number of cells located within the core of SOX9-expressing pancreatic epithelium (Fig. 2.1A-B). The βgal^+ cells at E12.5 express either glucagon (Fig. 2.1C) or insulin (Fig. 2.1D), indicating that they were early endocrine cells. βgal expression expands significantly between E12.5 and E14.5. At E14.5, differential βgal expression was detected in the $PDX1^+$ cells throughout the pancreatic epithelium (Fig. 2.1E). Epithelial cells with markedly lower βgal expression correspond to a subset of $PDX1^+$ cells that express SOX9 (Fig. 2.1F). At E15.5, high βgal expression was detected in the core of the differentiating pancreas (Fig. 2.1G-H, asterisks). These βgal^+ cells were positive either for glucagon or insulin (arrows in Fig. 2.1I and Fig. 2.1J respectively). At birth, βgal expression was detected in both islet and acinar cells (Fig. 2.1K-L, asterisk and arrow, respectively). Taken together, these expression data indicate that *Sel1l* is selectively expressed in the differentiating or differentiated pancreatic epithelial cells.

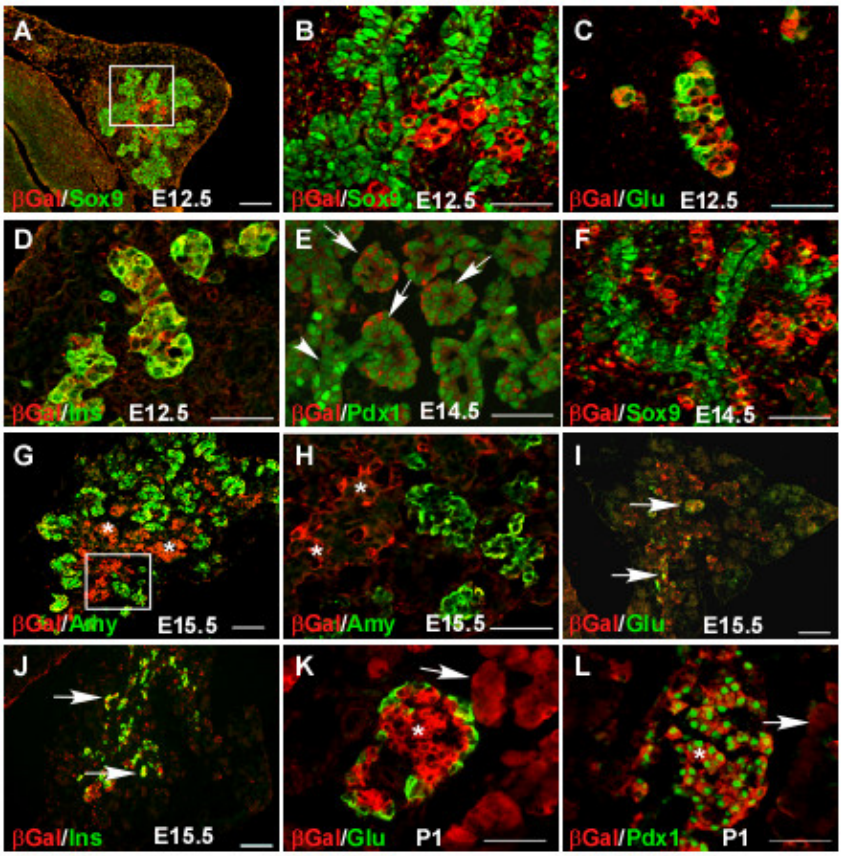

Figure 2.1 Spatiotemporal expression of *Sel1l* during development of the mouse pancreas. Pancreatic sections from timed Sel1l$^{+/\beta geo}$ embryos were co-immunostained with antibodies against βGal (red) and various pancreatic markers (green) as indicated on the left side of each panel. (B and H) Magnified views of the boxed area in A and G, respectively. (A-D) βGal expression at E12.5. βGal expression begins in a small number of cells within the core pancreatic epithelium at E12.5 (A). At this stage, there is no co-expression of βGal and SOX9 (B); however, βGal is co-expressed with either glucagon (C) or insulin (D). (E-F) βGal expression at E14.5. βGal is differentially expressed in the PDX1+ cells (E). While high βGal signal was detected in the epithelial branches (arrows), no or very low βGal signal was detected in the core "duct-like" epithelium (arrowhead). These core epithelial cells correspond to the subset of PDX+ cells that express SOX9 (F). (G-J) βGal expression at E15.5. βGal

expression increases markedly in the core pancreatic epithelium (G and H, asterisks). βGal co-localizes with either glucagon (I, arrows) or insulin (J, arrows). (K-L) βGal expression at postnatal day 1. βGal is expressed in both islet and acinar tissues (K, asterisk and arrow, respectively). βGal co-localizes with PDX1 in the islet tissue (L). Scale bars: (A, G, I and J) 50 μm; (B-F, K-L) 100 μm.

SEL1L deficiency results in defective pancreatic epithelial growth and branching morphogenesis

The gene trap in *Sel1l* contains a strong splicing acceptor signal that efficiently blocks splicing between exon 14 and 15, resulting in a truncated Sel1l transcript. Homozygous gene trap mice *(Sel1l*$^{\beta ge/\beta geo}$) exhibit systemic endoplasmic reticulum stress and die before E13.5 to E15.5 (Fig. 2.7). To determine if *Sel1l* is required for pancreatic epithelial growth, we performed morphometrical analysis of the developing pancreas of viable *Sel1l*$^{\beta geo/\beta geo}$ embryos. At E11.5, the dorsal and ventral pancreatic buds in *Sel1l*$^{\beta geo/\beta geo}$ embryos appeared to be closely linked (Fig. 2.2B), as compared to those in wild-type embryos (Fig. 2.2A). There was no significant difference in the pancreatic epithelial size between wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.2E). At E13.5, the dorsal pancreatic bud of *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.2C) was clearly smaller than that of wild-type embryos (Fig. 2.2F). In addition, while wild-type pancreatic epithelium displayed a well-branched structure, mutant pancreatic epithelium appeared to be a simple tube of epithelial cells. TUNEL assays revealed no significant increase of apoptosis in the developing pancreas of *Sel1l* mutant embryos (data not shown). Immunostaining using anti-PCNA (a cell proliferation indicator) antibody indicated that the pancreatic epithelium of *Sel1l* mutant embryos had a significantly lower rate of cell proliferation than wild-type pancreatic epithelium (Fig. 2.2G). Together, these results indicate that *Sel1l*, while dispensable for pancreatic

epithelial induction, is essential for the subsequent growth and branching morphogenesis of the pancreatic epithelial cells.

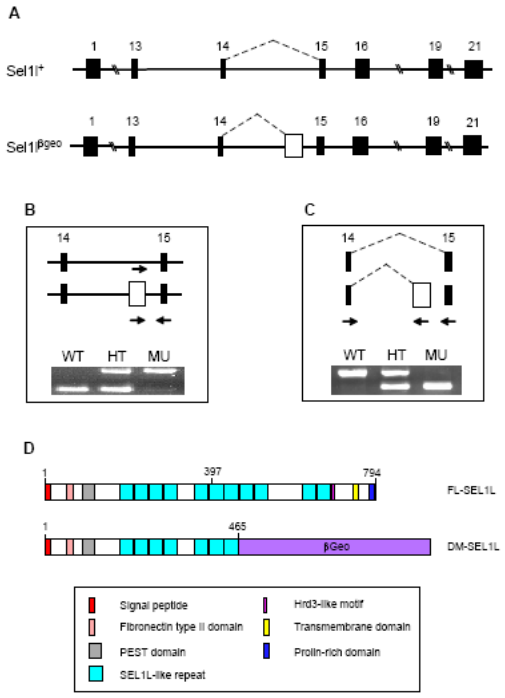

Figure 2.7 Characterization of the gene trap allele in *Sel1l*. (**A**) Schematic representation of the wild-type (*Sel1l*$^{+}$) and gene trap (*Sel1l*$^{\beta geo}$) allele in *Sel1l*. Solid black rectangles represent exons; the open box in the Sel1l^{-} allele represents the gene trap insertion. Splicing events are indicated by dashed lines. (**B**) PCR analysis of genomic DNAs from wild-type (WT), heterozygous (HT) and mutant (MU) mice using the indicated Sel1l and βgeo-specific primers (arrows). The data confirm the presence of a gene trap insertion in intron 14. (**C**) RT-PCR analysis of RNAs from E12.5 WT, HT and MU embryos using the indicated Sel1l and βgeo-specific primers (arrows). The data indicate that the gene trap allele in *Sel1l* completely blocks RNA splicing between exon 14 and 15, resulting in a truncated Sel1l transcript fused in frame with βgeo transcript. (**D**) Schematic representation of the full-length (FL) (top)

and deletion mutant (DM) SEL1L peptides generated from the wild-type and gene trap Sel1l alleles. Key protein domains are shown in colored boxes. Numbers represent amino acid positions. The gene trap allele in *Sel1l* generates a fusion protein containing the N-terminal 465 amino acids of SEL1L and βgeo. The mutant peptide lacks the Hrd3-like motif, the transmembrane domain, the proline-rich domain and 4 SEL1L-like repeats.

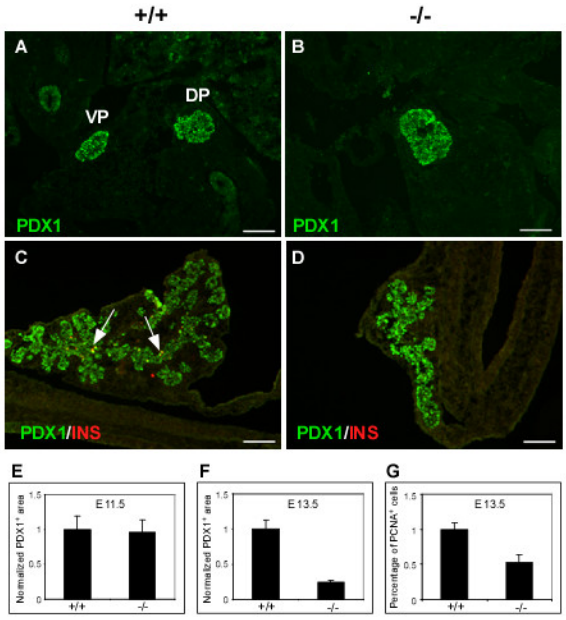

Figure 2.2 Impaired pancreatic epithelial growth and branching morphogenesis in Sel1l$^{\beta geo/\beta geo}$ embryos. (A-D) Immunohistological analysis of the developing pancreas of wild-type and Sel1l$^{\beta geo/\beta geo}$ embryos at E11.5 and E13.5; the genotypes of the pancreatic sections are indicated as +/+ and -/-, respectively. The following antibodies were used: Pdx1 (A-D, green) and insulin (C-D, red). (A-B) The dorsal and ventral pancreatic buds in Sel1l$^{\beta geo/\beta geo}$ embryos are fused together. (C-D) The dorsal pancreatic bud of Sel1l$^{\beta geo/\beta geo}$ embryos exhibits a markedly reduced epithelial size and an impaired branching morphology. (E-F) Statistical analyses of estimated pancreatic epithelial sizes of wild-type and Sel1l$^{\beta geo/\beta geo}$ embryos at E11.5 (E) and E13.5 (F).

Data were from three sets of wild-type and Sel1l$^{\beta geo/\beta geo}$ embryos. No significant difference in the epithelial sizes of wild-type and Sel1l-deficient pancreas at E11.5 was detected (E). At E13.5, the pancreatic epithelium of Sel1l-deficient embryos was significantly smaller (F). Scale bar: 100 μm.

SEL1L deficiency inhibits differentiation of acinar cells and significantly attenuates differentiation of endocrine cells

We next investigated the role of *Sel1l* in pancreatic epithelial cell differentiation. Since the first wave of cell differentiation occurs before E12.5 and the cells generated are mostly α-cells, we analyzed glucagon expression in E11.5 *Sel1l*$^{\beta geo/\beta geo}$ embryos. As shown in Fig. 2.3A-B and 2.3G, comparable numbers of glucagon$^+$ cells were observed in wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos. *Sel1l* is thus unlikely to be required for the generation of early endocrine cells during the first transition (E9.5 to E12.5).

While the majority of *Sel1l*$^{\beta geo/\beta geo}$ embryos (95%) die before the initiation of the major wave of cell differentiation in the developing pancreas (E13.5), about 5% of these embryos are viable at E15.5. To determine if *Sel1l* is required for differentiation of pancreatic epithelial cells during the secondary transition (E13.5 to E15.5), we analyzed the expression of three pancreatic lineage markers: insulin (β-cells), glucagon (α-cells) and amylase (acinar cells) in these embryos. Significant numbers of glucagon$^+$ and insulin$^+$ cells were detected in wild-type embryos at E15.5 (Fig. 2.3C and 2.3E). In contrast, the numbers of glucagon$^+$ and insulin$^+$ cells were significantly reduced in *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.3D and 2.3F, 2.3H and 2.3I). Abundant amylase$^+$ cells are present in wild-type embryos at E15.5 (Fig. 2.4E). In contrast, no amylase$^+$ cells are detectable in *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.4F). Disruption of *Sel1l* function thus results in an impaired differentiation of both acinar and islet cells.

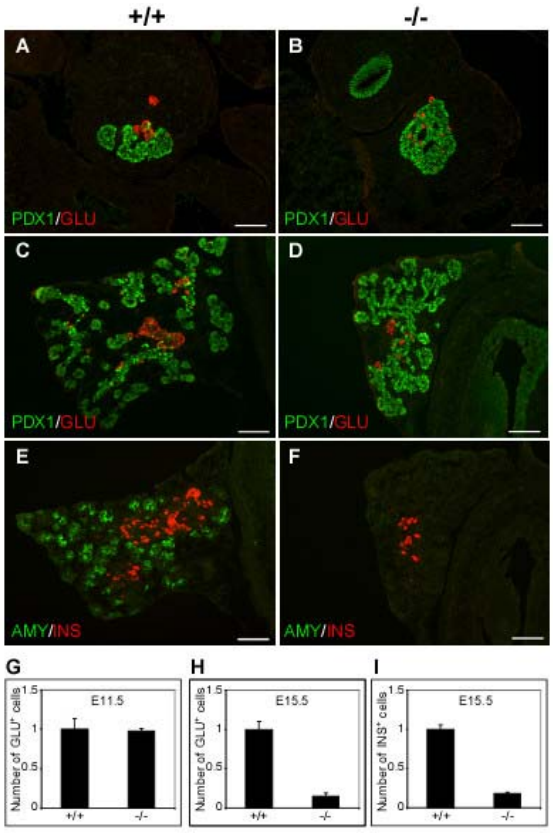

Figure 2.3 Inhibited endocrine and exocrine cell differentiation in *Sel1l*$^{\beta geo/\beta geo}$ embryos. (A-F) Immunohistological analysis of the developing pancreas of *Sel1l*$^{+/+}$ and *Sel1l*$^{\beta geo/\beta geo}$ embryos at E11.5 (A-B) and E15.5 (C-F); the genotypes of the pancreatic sections are indicated as "+/+" and "-/-", respectively. The following antibodies were used: Pdx1 (A-D, green), glucagon (A-D, red), insulin (E-F, red) and amylase (E-F, green). (A-B) At E11.5, Glu$^+$ cells were detected in the pancreatic epithelium of wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos in equal numbers. (C-D) At E15.5, the number of glucagon$^+$ cells was reduced in *Sel1l*$^{\beta geo/\beta geo}$ embryos as compared to wild-type control. (E-F) No amylase$^+$ cells were detected and the number of insulin$^+$ cells was significantly reduced in *Sel1l*$^{\beta geo/\beta geo}$ embryos. (G-I) Statistical analyses of the numbers of glucagon$^+$ and insulin$^+$ cells in wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos. Data were from three wild-type and three *Sel1l*$^{\beta geo/\beta geo}$ embryos. At E11.5, no

significant difference in the number of glucagon$^+$ cells in wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos was detected (G). The number of glucagon$^+$ and insulin$^+$ cells was markedly lower in *Sel1l*$^{\beta geo/\beta geo}$ embryos than in wild-type control embryos (H and I, respectively). Scale bar: 100 μm.

SEL1L-deficient pancreatic epithelium exhibits inhibited growth and differentiation *ex vivo*

To ensure that the growth and differentiation defects observed in *Sel1l*$^{\beta geo/\beta geo}$ embryos were not due to the effect of global embryonic growth retardation of these embryos, we studied the pancreatic phenotype of *Sel1l*$^{\beta geo/\beta geo}$ embryos using an organ culture system. The dorsal pancreatic bud of wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos were isolated at E11.5 and cultured for 8 days. The cultured pancreatic explants were then analyzed by immunofluorescence using antibodies against PDX1 and insulin. In general, *Sel1l*-deficient pancreatic epithelium grows poorly (Fig. 2.8B and 2.8D) with about half of the cultured mutant epithelia failing to form branched epithelial structures. This is in sharp contrast to the growth behavior of wild-type pancreatic epithelia, which form a branched epithelial structure (Fig. 2.8). The number of insulin$^+$ cells was significantly decreased in *Sel1l*-deficient pancreatic explants (Fig. 2.4B), as compared to that in wild-type control explants (Fig. 2.4A). Amylase$^+$ cells were detected in cultured *Sel1l*-deficient explants (Fig. 2.4D), although the number of amylase$^+$ cells was significantly lower compared to that in wild-type control explants (Fig. 2.4C). Thus, the pancreatic epithelium of *Sel1l*$^{\beta geo/\beta geo}$ embryos displays impaired growth, branching morphogenesis and lineage differentiation *ex vivo*. These findings confirm that the pancreatic defects observed in *Sel1l*-deficient embryos at E15.5 are not due to the global growth retardation.

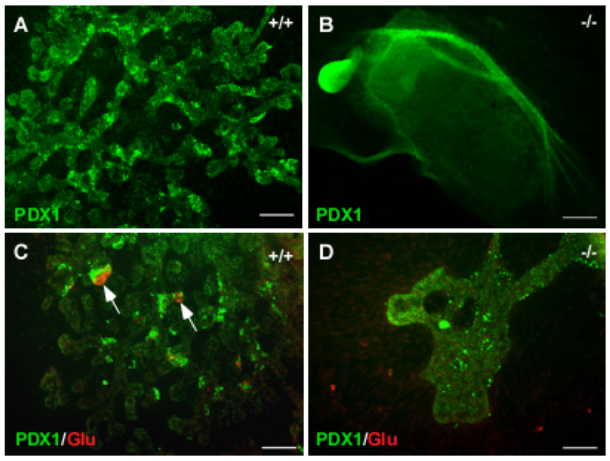

Figure 2.8 *Sel1l*-deficient pancreatic epithelium exhibits impaired growth, branching morphology and differentiation *ex vivo*. The dorsal pancreatic bud of wild-type (+/+) and *Sel1l*$^{\beta geo/\beta geo}$ (-/-) embryos were isolated at E11.5 and cultured as described in Materials and Methods. Cultured pancreatic explants were immunostained with the indicated antibodies. *Sel1l*-deficient pancreatic epithelium grows poorly and displays impaired branching morphology (**B** and **D**), as compared to wild-type control explants (**A** and **C**). Scale bar: 100 μm.

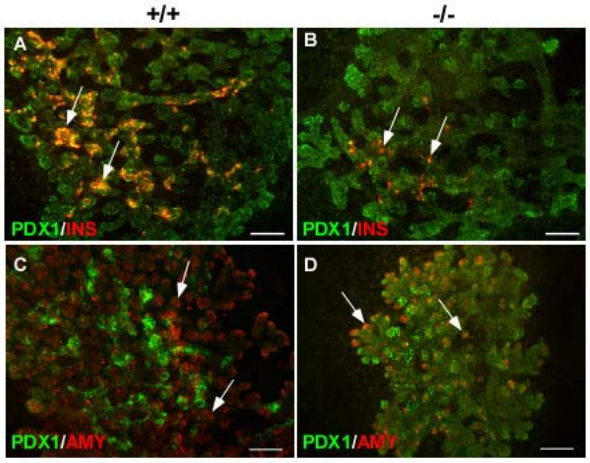

Figure 2.4 *Sel1l*-deficient pancreatic epithelium exhibits impaired growth, branching morphology and differentiation *ex vivo*. Dorsal pancreatic buds of wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos were isolated at E11.5 and cultured as described in

Materials and Methods. The genotypes of the pancreatic explants are indicated as "+/+" and "-/-", respectively. Cultured pancreatic explants were immunostained using the indicated antibodies. *Sel1l*-deficient pancreatic epithelium grows poorly and displays impaired branching morphology (B and D), as compared to wild-type control explants (A and C). The number of insulin+ cells in mutant pancreatic epithelia were markedly reduced (B, arrows), as compared to the wild-type controls (A, arrows). Amylase expression was detected in *Sel1l*-deficient pancreatic epithelium (D, arrows), but the expression is significantly lower than that in wild-type epithelium (C, arrows). Scale bar: 100 μm.

Pancreatic progenitor cells in SEL1L-deficient embryos at E15.5 are confined to a pluripotent progenitor state

To gain insight into the molecular basis underlying the pancreatic defects in *Sel1l*$^{\beta geo/\beta geo}$ embryos, we first analyzed the expression of several transcription factors important for pancreatic epithelial growth and lineage formation. Immunostaining was carried out to assess the expression of *Sox9, Pdx1* and *Ptf1a* in E15.5 *Sel1l*$^{\beta geo/\beta geo}$ embryos. PDX1 expression was uniformly detected in the pancreatic epithelial cells of *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.5B). This is in sharp contrast to wild-type embryos where PDX1 expression was differentially expressed in the pancreatic epithelial cells, with high expression in a subset of the core epithelial cells and low expression in the peripheral epithelial cells (Fig. 2.5A, white arrow and red arrowheads, respectively). No PTF1a expression was detected in the pancreatic epithelium of *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.5B), whereas clear co-expression of PTF1a and PDX1 was detected in the proacini in the periphery of the pancreatic epithelial buds (Fig. 2.5A, white arrows). SOX9 was detected in approximately 20% of the PDX1^{+} cells in wild-type embryos (Fig. 2.5C, red arrows). In contrast, SOX9 expression was observed in more than 70%

of the PDX1^{+} cells in *Sel1l*$^{\beta geo/\beta geo}$ embryos (Fig. 2.5D, asterisk). These findings are consistent with the notion that the pancreatic epithelial cells in *Sel1l*$^{\beta geo/\beta geo}$ embryos at E15.5 are restricted the pancreatic progenitor state.

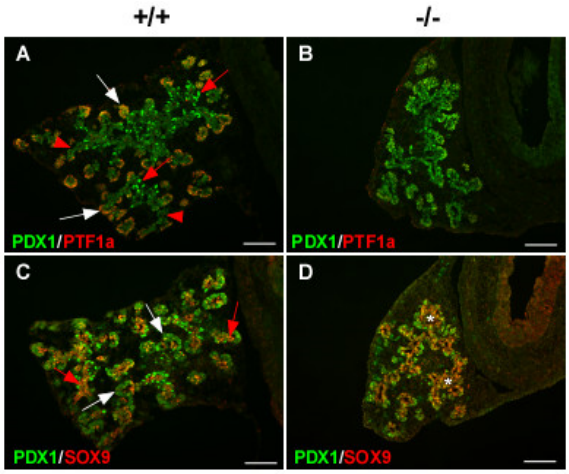

Figure 2.5 *Sel1l*-deficient pancreatic epithelial cells fail to commit to lineage precursors during the secondary transition. (A-D) Immunohistological analysis of the developing dorsal pancreas of wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos at E15, using the indicated antibodies. The genotypes of the pancreatic sections are indicated as "+/+" and "-/-", respectively. (A-B) PTF1a is expressed in the periphery of wild-type pancreatic epithelium marking the commitment of pancreatic epithelial cells into the exocrine lineage (A, white arrows); PTF1a expression is absent in the mutant pancreas (B). Up-regulation of PDX1 expression in a subset of epithelial cells in the core of wild-type pancreatic epithelium marks the commitment of pancreatic epithelial cells into the endocrine lineage (A, red arrows); whereas PDX1 is uniformly expressed in mutant pancreatic epithelial cells (B). (C-D) SOX9 is expressed in a small population of epithelial cells in wild-type pancreatic epithelium (C, red arrows); in contrast, SOX9 mostly co-localizes with PDX1 in mutant pancreatic epithelium (D, asterisks). Scale bar: 100 μm.

Pharmacological inhibition of Notch signaling rescues endocrine lineage formation in Sel1l$^{\beta geo/\beta geo}$ embryos

Sel1l was thought to be a negative regulator for Notch signaling [47]. Indeed, the pancreatic phenotype in *Sel1l*$^{\beta geo/\beta geo}$ embryos shows remarkable similarity to that of mouse or zebrafish embryos over-expressing Notch intracellular domain, the constitutively active form of Notch receptors [26, 28, 50]. We speculated that the impaired pancreatic epithelial growth and differentiation in Sel1l-deficient embryos may be due to an increased Notch signaling activity. To test this possibility, we used the γ-secretase inhibitor, DAPT (Difluorophenacetyl-al-alanyl-S-phenylglycine-t-butyl ester), to suppress Notch signaling in pancreatic explants culture. The dorsal pancreatic bud of wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos was isolated at E11.5 and cultured for 8 days in the absence or presence of DAPT. At a concentration of 10 μM, DAPT did not exhibit detectable effects on the growth, branching morphology and endocrine cell differentiation of wild-type pancreatic epithelium (Fig. 2.6A-B and 2.6E). At the same concentration, however, DAPT caused a significant expansion of the SEL1L-deficient pancreatic epithelium (Fig. 2.6C-D). Importantly, DAPT treatment resulted in a marked increase in the number of insulin$^+$ cells (Fig. 2.6D and 2.6F). These observations indicate that pharmacological inhibition of Notch signaling rescues, at least partially, the pancreatic epithelial growth and endocrine differentiation defects of *Sel1l*$^{\beta geo/\beta geo}$ embryos.

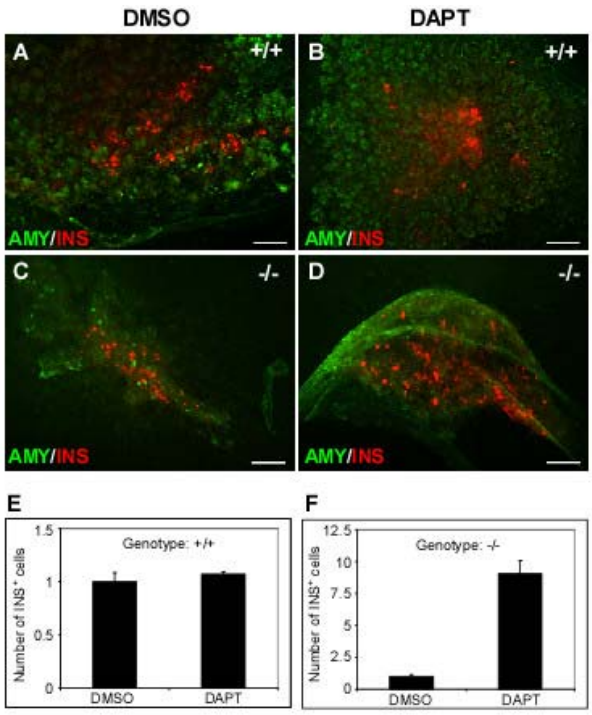

Figure 2.6 DAPT treatment rescues the pancreatic phenotype of *Sel1l*$^{\beta geo/\beta geo}$ embryos. The dorsal pancreatic bud of wild-type (+/+) and *Sel1l* mutant (-/-) embryos was isolated at E11.5 and cultured for 8 days in the presence of 0.1% DMSO (control) and 10 μM of DAPT. (A-D) Immunohistological analysis of DAPT-treated and non-treated pancreatic buds with the indicated antibodies. (E-F) Quantification of insulin^{+} cells in DMSO and DAPT-treated pancreatic explants. DAPT did not significantly change the number of insulin^{+} cells in wild-type pancreatic buds (A-B and E), however, it induced a marked increase of insulin^{+} cells in *Sel1l* mutant pancreatic buds (C-D and F). Scale bar: 100 μm.

2.4 Discussion

The genetic determinants underlying vertebrate pancreatic development are not completely understood. In the present study, we report that *Sel1l* expression in the developing mouse pancreas coincides with differentiation of the endocrine and exocrine lineages during the second transition. Embryos homozygous for a gene trap insertion in *Sel1l* display impaired pancreatic epithelial growth and differentiation. Pharmacological inhibition of Notch signaling partially rescues the pancreatic phenotype of SEL1L-deficient embryos. Together, these data provide evidence that *Sel1l* is required for growth and differentiation of the mammalian pancreatic epithelial cells. The underlying mechanism may be that *Sel1l* functions as a negative regulator for the Notch signaling pathway by facilitating degradation of the Notch intracellular domain.

Donoviel *et. al*. previously reported that the mouse *Sel1l* gene is highly transcribed in the acini of the developing pancreas at E14.5 and E17.5 [41]. We show, by immunohistological analysis of the β-galactosidase reporter in *Sel1l*, that *Sel1l* is also highly expressed in early glucagon$^+$ and insulin$^+$ cells (Fig. 2.1C and 2.1D, respectively) generated during the first transition period of pancreatic development [5, 51, 52]. Consistent with the *Sel1l* mRNA expression pattern reported by Donoviel *et. al*., we reveal that at E14.5 *Sel1l* is broadly expressed in the PDX1$^+$ pancreatic epithelia (Fig. 2.1E). Interestingly, significantly lower *Sel1l* expression is observed in the undifferentiated pancreatic epithelial cells (Fig. 2.1F) that express SOX9 [10, 11]. Our findings therefore indicate that *Sel1l* expression is enhanced in the committed pancreatic epithelial cells or differentiated endocrine and exocrine cells.

The selective expression of *Sel1l* in differentiating or differentiated pancreatic epithelial cells is highly suggestive of a role for *Sel1l* in promoting lineage differentiation during the secondary transition. To define the role of *Sel1l* in pancreatic

development, we characterized the pancreatic phenotype of embryos homozygous for a gene trap insertion in *Sel1l*. We show that wild-type and *Sel1l*-deficient embryos have a comparable pancreatic epithelial size at E11.5 (Fig. 2.2A-B and 2.2E), suggesting that *Sel1l* function is dispensable for the induction and early growth of pancreatic epithelial cells. At E13.5, however, *Sel1l*-deficient pancreatic epithelia are significantly smaller and display an impaired branching morphology (Fig. 2.2C-D and 2.2F). Importantly, *Sel1l*-deficient pancreatic epithelia at E15.5 completely lack amylase$^+$ cells (Fig. 2.3F) and have significantly reduced numbers of glucagon$^+$ and insulin$^+$ cells (Fig. 2.3D and 2.3F, respectively). Also, by analyzing the phenotype of cultured *Sel1l*-deficient dorsal pancreatic epithelia, we confirmed that the pancreatic defects in *Sel1l*-deficient embryos are not due to the effect of global growth retardation of the *Sel1l*-deficient embryos (Fig. 2.4A-D). Together, these data strongly suggest that *Sel1l* plays an important role in mouse pancreatic organogenesis by facilitating differentiation of the endocrine and exocrine lineages of the pancreas.

The *C. elegans* ortholog of the mammalian *Sel1l* gene, *Sel-1*, was originally identified by genetic screening as a negative regulator for lin-12/Notch signaling [44, 53]. Biochemical studies have shown that *Sel-1* regulates lin-12/Notch receptor turnover [45, 46]. Whether or not Sel1l has similar functions in the mammalian system remains unclear [54]. In this study, we provide two lines of evidence that suggest *Sel1l* also functions as a negative regulator for Notch signaling, at least in the developing mouse pancreas. First, the observed pancreatic phenotype in *Sel1l* mutant embryos show remarkable similarity to the pancreatic phenotype of transgenic vertebrate embryos over-expressing the Notch intracellular domain, the constitutively active form of Notch [26, 28, 50]. Second, we show that treatment of cultured *Sel1l*-deficient pancreatic epithelium with DAPT, a potent Notch signaling inhibitor, partially rescues

the growth and differentiation defects of *Sel1l*-deficient pancreatic epithelium (Fig. 2.6D and 2.6F).

How does SEL1L negatively control Notch signaling in the developing mammalian pancreas is the focus of our current investigation. Genetic and biochemical evidences from other organisms such as *Drosophila* and *C. elegans* suggest that Notch signaling is regulated at multiple levels and through multiple mechanisms, including stochastical gene expression [34], selective ligand-receptor interactions [35], intracellular trafficking [37], and stability of NICD, the active form of Notch receptors [30]. Given the previous finding that mammalian NICD are ubiquitinated and degraded through both the proteasomal and lysosomal systems [55, 56], it is conceivable that Sel1l may be directly or indirectly involved in control of NICD degradation. In this regard, it is noteworthy to mention that recent biochemical data from our laboratory indicate that NICD has significantly higher protein stability in *Sel1l*-deficient MEF cells (data not shown). Further proof of this hypothesis will require a detailed biochemical analysis of SEL1L and Notch interactions.

In addition, biochemical evidences from other laboratories have underscored the importance of *Sel1l* in maintaining ER homeostasis. SEL1L interacts with the E3 ubiquitin ligase HRD1 to facilitate the dislocation of unfolded or misfolded proteins from the ER lumen into the cytosol for degradation [48, 49, 57-60]. Consistent with this, we have recently shown that homozygous *Sel1l* mutant mice develop systemic ER stress. The impact of ER stress on pancreatic epithelial proliferation and differentiation remains undefined from the current study. However, several hypothetical roles of ER stress in pancreatic organ development and growth can be proposed. First, ER stress affects global gene expression. Upon ER stress, translation is generally down to reduce production of ER client proteins. Concomitantly, transcription of genes encoding ER chaperones and ER-associated degradation (ERAD)

machinery is increased. Second, ER stress blocks or reduces protein secretion by mammalian cells. This will directly affect cell signaling and cell functions. Finally, prolonged ER stress activates the signaling pathways leading to apoptosis.

2.5 Conclusions

We report that during mouse pancreatic development, *Sel1l* is preferentially expressed in the differentiating or differentiated pancreatic epithelial cells. Disruption of *Sel1l* function results in impaired endocrine lineage formation and delayed exocrine lineage differentiation. Pharmacological suppression of Notch signaling in cultured pancreatic explants partially rescues the endocrine cell differentiation defect. Our data suggest that *Sel1l* may regulate pancreatic epithelial growth and differentiation by suppressing Notch-mediated signaling.

2.6 Methods

Sel1l gene trap mice

The chimeric founder mouse used to generate S*el1l* gene trap mice was generated by microinjection of a commercially available mouse embryonic stem (ES) cell clone, CA0017, into C57/BL6 blastocysts. This ES cell clone contains a gene trap insertion in the 14th intron of the *Sel1l* gene. All mice or mouse embryos were genotyped by PCR analysis of tail or toe genomic DNA using the following PCR primers:

F-Sel1l: 5'-TGGGACAGAGCGGGCTTGGAAT-3';

R-Sel1l: 5'-CACCAGGAGTCAAAGGCATCACTG-3';

R-βGeo: 5'ATTCAGGCTGCGCAACTGTTGGG-3'.

All animal experiments were performed in accordance with the Cornell Animal Care and Use Guidelines.

Histology and immunohistochemistry

Embryos or pancreatic rudiments were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 12 hours or longer. PFA-fixed specimens were equilibrated in 30% sucrose in PBS at 4°C and embedded in O.C.T. Sections were cut at 10 μm and immunostained essentially as described [61, 62]. Briefly, tissue sections were permeabilized with 0.2% Triton X-100 in PBS for 20 min, washed 3 times in 0.1% Triton X-100 in PBS and 3 times in PBS. The permeabilized sections were pre-incubated with 5% normal donkey serum and 1% BSA in PBS at room temperature for at least 1 hour, followed by incubation in the same solution with primary antibodies at 4°C overnight. The antibody-bound sections were then washed three times in 0.1% Triton X-100 in PBS, 3 times in PBS and then incubated with secondary antibodies for 1-3 hrs at room temperature. Primary antibodies were diluted in 1% BSA in PBS as follows: rabbit anti-b-Galactosidase (Immunology Consultants Laboratory), 1:500; guinea pig anti-Pdx-1 (Abcam), 1:1000; goat anti-Pdx1 (Abcam), 1:1000; guinea pig anti-insulin (Linco), 1:1000; rabbit anti-glucagon (Covance), 1:500; goat anti-glucagon (AbD Serotec), 1:200; rabbit anti-Sox9 (gift from Dr. Michael Wegner), 1:1000; rabbit anti-Ptf1a (gift from Dr. Helena Edlund), 1:1000); and rabbit anti-amylase (Sigma), 1:500. Secondary antibodies were diluted in 1% BSA in PBS as follows: donkey anti-rabbit IgG conjugated to Cy3 (Jackson Immuno Research), 1:1000; donkey anti-guinea pig IgG conjugated to Cy2 (Jackson Immuno Research), 1:500. Fluorescence images were acquired using an Axiovert 40 microscope (Zeiss) equipped with an AxioCam camera.

Pancreatic morphometry and cell counting

Quantitative morphometry of the developing pancreas and cell counting were performed essentially as described [11]. Briefly, pancreata of age-matched wild-type and *Sel1l*$^{\beta geo/\beta geo}$ embryos were sectioned through and every fifth section was

immunostained with anti-PDX1 or co-immunostained with anti-PDX1 and anti-Glucagon. Quantification of pancreatic epithelium area ($PDX1^+$ area) was performed using AxioVision Imaging analysis software (version 4.6.3). The data were presented as averages ± SEM (μm^2) of three independent pancreata. Statistical analysis was performed using the Student's two-sample *t* test and significance is regarded as $p \leq 0.05$.

Pancreatic organ culture and DAPT treatment

Sel1l$^{+/\beta geo}$ mice were intercrossed to generate embryos of defined genotypes: wild-type (*Sel1l*$^{+/+}$), heterozygous (*Sel1l*$^{+/\beta geo}$) and homozygous (*Sel1l*$^{\beta geo/\beta geo}$). The day of the vaginal plug was taken as embryonic (E) day 0.5. Pancreatic bud dissection and culture were performed essentially as described [63]. Briefly, E11.5 embryos were decapitated and the dorsal pancreatic rudiment together with surrounding mesenchymal tissue was removed in PBS. The dissected pancreatic buds were rinsed once in culture medium, transferred to a fibronectin-coated 8-well LabTek chamber slide with the epithelial side touching the bottom of the slide and cultured for 72 hours in basal medium with Earle's salts (Gibco 21010-046), 1 × glutamine, 10% FBS and antibiotics. The cultured pancreatic rudiments were fixed individually for 30 minutes in 4% paraformaldehyde in PBS for immunohistological analysis. DAPT treatment of cultured pancreatic explants was performed essentially as described [64]. DAPT stock solution was prepared at a concentration of 10 mM in DMSO and was aliquoted into single-usage aliquots. For Notch inhibition experiments, pancreatic buds were cultured in complete medium containing 10 μM of DAPT. Control pancreatic buds were cultured in complete medium containing 0.1% DMSO. The DMSO or DAPT-treated pancreatic buds were briefly fixed and analyzed by immunostaining.

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CHAPTER 3

RFX6 FUNCTIONS AS A TRANSCRIPTION REGULATOR FOR NKX6.1 IN PANCREATIC β CELLS

3.1 Abstract

Background: The vertebrate pancreas is comprised of endocrine, exocrine and ductal cells. These cells derive from a common pool of pancreatic progenitors during embryonic development. Understanding the genetic determinants underlying normal pancreatic development will accelerate the development of cell-based therapies for the treatment of diabetes mellitus.

Methodology/Principal Findings: Through differential gene expression screening, we have identified a novel mouse gene, regulatory factor x 6 (Rfx6), with islet-specific expression. We show here that the putative RFX6 protein contains a highly conserved winged helix-turn-helix DNA-binding domain (DBD). Ectopically expressed RFX6 is predominantly localized in the nucleus of mammalian cells. Knocking down of Rfx6 function using siRNA in the pancreatic β-cell line βTC3, causes down-regulation of Nkx6.1 mRNA expression, while ectopic expression of Rfx6 stimulates Nkx6.1 promoter activity. Zebrafish embryos injected with Rfx6-specific morpholino antisense oligos exhibit attenuated insulin expression.

Conclusions: Taken together, these results strongly suggest that RFX6 functions as a transcriptional regulator for Nkx6.1 and may be involved in regulating β-cell differentiation and growth.

3.2 Introduction

The vertebrate pancreas contains acinar, ductal and islet cells. These specialized cells derive from a transient pool of multipotent progenitor cells during

embryonic development [1]. In mice, pancreatic development begins at about embryonic day 8.5-9 (E8.5-9) with the formation two pancreatic epithelial buds. During the next several embryonic days (E9 to E12.5), the pancreatic epithelium grows and branches out to form a complex epithelial structure consisting of numerous elongated tubules [2, 3]. This period of epithelial growth creates a transient pool of pluripotent pancreatic progenitors that later differentiate to give rise to all the specialized cell types of the adult pancreas, including ducts, acini and islets [4, 5], which include glucagon-secreting α-cells, insulin-
secreting β-cells, somatostatin-secreting δ-cells and pancreatic polypeptide-secreting pp¬cells [1].

Early pancreatic epithelial cells express PDX1 [6, 7], SOX9 [8, 9] and PTF1a [5]. Genetic mutations in these genes impair early pancreatic epithelial expansion and morphogenesis and, therefore, prevent formation of the islets, acini and ducts of the pancreas. Mice lacking PDX1 have no pancreas; the pancreatic epithelium fails to grow and to undergo branching morphogenesis and there is no pancreatic formation [10-12]. The pancreatic epithelium of mice with targeted disruption of PTF1a grows poorly [13] and adopt the normal fate of duodenal epithelium [5]. SOX9 expression is restricted to the mitotically active multipotent pancreatic progenitors and pancreas-specific inactivation of Sox9 results in premature differentiation of the pancreatic epithelial cells [8, 9]. These findings suggest that PDX1, PTF1a and SOX9 coordinate the induction and growth of the early pancreatic epithelium.

During mouse pancreatic organogenesis, endocrine cells arise in two temporally distinct phases. The first sign of endocrine differentiation is the appearance of a small group of (mostly glucagon-expressing cells) endocrine cells in the primitive pancreatic epithelial buds from E9 to E12.5 (primary transition) [14, 15]. These early glucagon-expressing cells display a molecular profile that is distinct from mature

alpha cells [16] and do not contribute significantly to the mature endocrine mass [17, 18]. Between E13.5 to E15.5, endocrine cells of all subtypes along with exocrine cells are produced in significant numbers (secondary transition) [19, 20]. Genetic inactivation of a single transcription factor, Neurogenin-3 (NGN3), abolishes the formation of all the endocrine cell types in mice [21]. Conversely, forced expression of NGN3 in early PDX1+ epithelial cells induces endocrine cell formation [22, 23]. Together, these results suggest that NGN3 is both essential and sufficient for initiation of endocrine cell differentiation in the developing pancreas.

Significant efforts have been made to understand the roles of other transcriptional factors, including NeuroD1 [24, 25], Pax4 [26], Pax6 [27], Nkx2.2 [28], Nkx6.1 [29], Arx [30], Isl1 [31] and Insm1 [32]. It is now generally accepted that, during pancreatic organogenesis, a network of transcription factors coordinate pancreatic morphogenesis by functioning in concert to restrict the developmental potentials of the pancreatic progenitors in a spatial and stage-specific manner [20, 33-36].

The regulatory factor x (RFX) family of transcriptional factors comprises five structurally related members (RFX1-5) [37]. RFX proteins share a highly conserved 76¬amino acid winged helix-turn-helix DNA binding domain (DBD). With the exception of RFX5, RFX proteins also contain three additional conserved regions: a dimerization domain (DMD), B and C domains [37]. RFX proteins can form homo- or heterodimers and regulate the expression of their target genes by binding to the symmetric X-box motif (5'-GTNRCC(0-3N)RGYAAC-3', where N is any nucleotide, R is a purine and Y is a pyrimidine) in the promoter region [38]. The developmental and physiological roles of the Rfx genes have just begun to be recognized. Rfx3 is expressed in the ciliated cells of the node and targeted disruption of Rfx3 in mice causes left-right asymmetry defects [39]. Recently, it has been shown that Rfx3

deficiency also causes a significant reduction of beta cell mass and disorganization of the pancreatic islets [40]. Rfx4 is distantly related to Rfx3 and disruption of a brain-specific isoform of RFX4 (RFX4_v3) causes fatal failure of dorsal midline brain structure formation [41]. The functions of RFX1, RFX2 and RFX5 remain elusive, but they have been implicated in regulating the expression of several genes involved in immune responses, such as interferon gamma and class II major histocompatability complex (MHC) genes [42-44].

Rfx6 is a recently identified member of the mammalian Rfx gene family [45]. Among all the RFX proteins, RFX6 shows the highest sequence and structural homology to RFX4. Through differential gene expression screening, we have found that the mouse Rfx6 gene is preferentially expressed in the islet cells of the adult pancreas. In this study, we have analyzed the sequence, expression and functions of RFX6. Our findings indicate that RFX6 functions as a transcriptional activator for the endocrine lineage specific gene Nkx6.1. These data, together with the recently published mouse genetic data [46, 47], suggest that RFX6 is an important regulator for the development and function of the mammalian endocrine pancreas.

3.3 Results

Identification of the mouse Rfx6 gene

In an attempt to identify novel factors with enriched expression in pancreatic islets, we screened 30 candidate genes using semi-quantitative RT-PCR. These genes were selected based on their expressed sequence tag (EST) profiles in the Unigene database [48]. One of the genes found to be preferentially expressed in the mouse pancreatic islets was previously named Rfxdc1 (for regulatory factor X domain-containing 1). Recently, Rfxdc1 has been re-named as Rfx6 (for regulatory factor X 6, GeneID#: 320995) based on its significant sequence homology with other members of

the RFX family of regulatory proteins [45]. Twenty four Rfx6-related mouse expressed sequence tags (EST) are currently registered in the GenBank database. These EST sequences predict at least two Rfx6 transcripts: Rfx6-v1 (NM_001159389) and Rfx6-v2 (NM_177306). The longest Rfx6 mRNA has a putative ORF of 2793 nucleotides which encodes a peptide of 893 amino acids (Accession # FJ804414). The deduced peptide shows 87.4, 78.8 and 65.2 percent sequence identity to the predicted human, chicken and zebrafish RFX6, respectively. The N-terminal half of the RFX6 sequence is highly conserved among human, mouse and zebrafish. The conserved region contains a winged helix-turn-helix DNA-binding domain (DBD), a dimerization domain (DMD) and two other domains, B- and C-domains (Fig.1, green, blue, pink and yellow box, respectively). These domains are found in members of the RFX family of transcriptional factors [45]. RFX6 shows 28.5, 29.2, 27.8, 36.6 and 12.7 percent sequence identity to RFX1 (NM_009055), RFX2 (NM_009056), RFX3 (NM_011265), RFX4 (NM_001024918) and RFX5 (NM_017395), respectively. Predictions of protein sorting and other sequence motifs using the online PSORT software [49] failed to reveal any nuclear localization signal in RFX6, but identified 7 highly conserved dileucine motifs within the C- and DMD-domains (Fig. 3.1, highlighted in red).

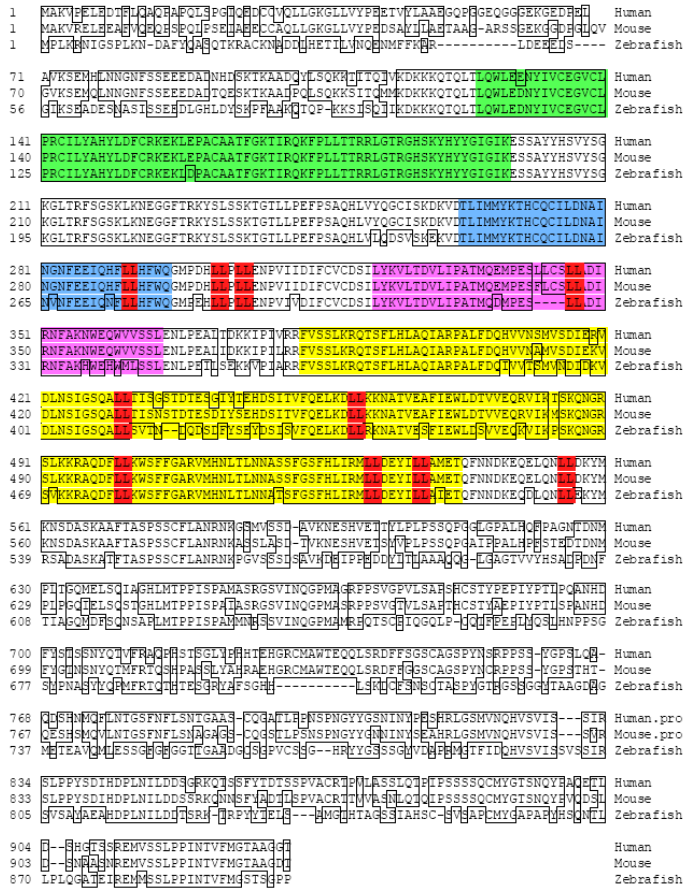

Figure 3.1 RFX6 sequence is highly conserved among vertebrate species. Human, mouse and zebrafish RFX6 sequences (BC039248, FJ804414 and XM_679861, respectively) were aligned using the Lasergene Megalign program. Note that the N-terminal half of the RFX6 sequence is highly conserved among human, mouse and zebrafish. The conserved region contains a winged helix DNA-binding (green), B- (blue), C- (pink) and dimerization (yellow) domains. Seven conserved dileucine motifs are found in RFX6 (highlighted in red).

The mouse Rfx6 gene is preferentially expressed in the developing and matured pancreatic islets

According to the gene expression data available from UniGene (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.109190)

and UCSC Mouse Gene Sorter (http://genome.ucsc.edu/cgi¬bin/hgNear?hgsid=150478630&org=Mouse&db=mm9&near_search=rfxdc1&submit=Go%21&near_order=expGnfAtlas2&near.count=100), Rfx6 is preferentially expressed in the endocrine compartment of the pancreas. To assess the tissue distribution of Rfx6 transcripts in adult mice, we isolated total RNAs from brain, liver, lung, kidney, skeletal muscle, heart, whole pancreas and islets of 6 week old female mice and performed quantitative RT-PCR analysis. As shown in Fig. 3.2A, Rfx6 transcripts were detected exclusively in the islets of Langerhans of the pancreas. To assess the expression of the mouse Rfx6 gene during pancreatic development, we isolated total RNA from the dorsal pancreatic bud and two adjacent tissues, stomach and spleen rudiments, from E14.5-15.5 embryos and performed semi-quantitative RT-PCR analysis. Significantly higher Rfx6 mRNA was detected in the dorsal pancreatic bud than in the stomach and spleen rudiments [Fig. 3.2B]. Together, these results indicate that the mouse Rfx6 gene is preferentially expressed in the developing pancreas and in the endocrine lineage of the matured pancreas in mice.

RFX6 is preferentially localized in the nucleus of mammalian cells

The predicted RFX6 peptide sequence contains a winged helix DNA-binding domain but has no obvious nuclear localization signal. Previous studies showed that RFX1-5 functions as transcriptional regulators in various embryonic and adult tissues. To determine whether RFX6 is nuclear factor, we assessed the sub-cellular localization of RFX6 protein. For this study, Hela cells were transfected with an expression plasmid encoding a FLAG-tagged RFX6. The transfected cells were immunostained with an anti-FLAG antibody. Of the transfected Hela cells, 55.3% exhibit FLAG-RFX6 in the nucleus (Fig. 3.3A and D), 26.3% contain FLAG-RFX6 in the nucleus (Fig. 3.3B and D) and the remainder cells show FLAG-RFX6 in both the

nucleus and the cytoplasm (Fig. 3.3C). Thus, ectopically expressed RFX6 is preferentially localized in the nucleus of mammalian cells. Given the fact that RFX6 contains a highly conserved winged-helix DNA-binding domain, these results suggest that RFX6 may function as a transcriptional regulator.

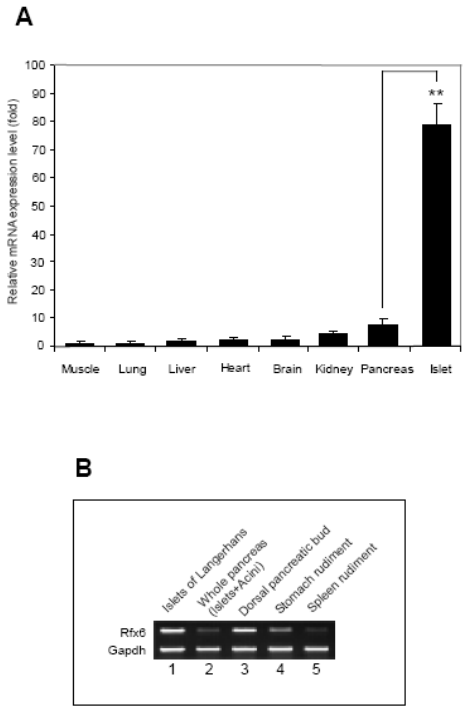

Figure 3.2 Rfx6 mRNA is highly expressed in the developing and matured pancreas and in islets of Langerhans of the pancreas. (A) Quantitative RT-PCR of Rfx6 mRNA expression in adult tissues. Tissue-specific RNAs were isolated from 6 week old female mice using Trizol. Rfx6 mRNA expression was analyzed by quantitative RT-PCR. The mRNA expression of 18S was used as an internal control to normalize Rfx6 expression as described in Materials & Methods. The value of Rfx6 mRNA expression in skeletal muscle was set to 1 and used to calculate relative expression in other tissues. The data were expressed as means + S.D. Statistical analysis was performed using the Student's t-test and $p < 0.05$ is considered significantly different. (B) Semi-quantitative RT-PCR analysis of Rfx6 mRNA

expression in islets of Langerhans, whole pancreas, pancreatic, stomach and spleen rudiments. Whole pancreas and islets of Langerhans were isolated from 6 week old female mice. Dorsal pancreatic buds, stomach and spleen rudiments were dissected from E13.5-15.5 wild-type mouse embryos. Total RNAs were isolated from pooled pancreas, stomach and spleen rudiments and Rfx6 mRNA expression was analyzed by RT-PCR. Gapdh mRNA expression across the tissues was used as an internal control to ensure the use of equal amount of starting RNA.

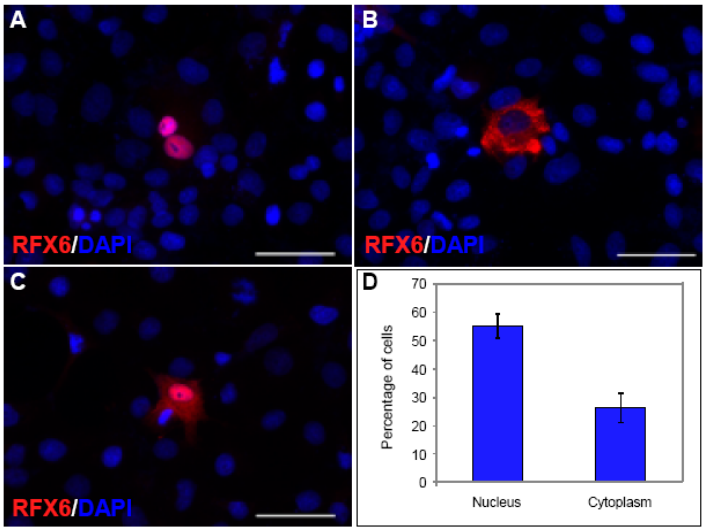

Figure 3.3 RFX6 is exclusively localized in the cytoplasm (A-C) Representative images of immunostained Hela cells ectopically expressing RFX6. Hela cells were transfected with an expression plasmid encoding Flag-tagged RFX6. Twenty four hour after transfection, Hela cells were briefly fixed in 4% PFA and immunostained with an anti-FLAG antibody (Red). Nuclei were stained with DAPI (blue). RFX6-FLAG fusion protein is observed in the cytoplasm (A), nucleus (B) or both compartments (C). (D) Quantification of the percentage of cells with cytoplasmic, nucleus and mixed localizations. Scale bars: 50 μm.

RFX6 functions as a transcriptional regulator for the endocrine lineage specific gene *Nkx6.1*

Previous studies have identified and characterized more than a dozen of endocrine lineage-specific transcription factors. These factors are involved in control of insulin gene expression, β-cell function and growth. To assess whether RFX6 plays any role in adult β cells, we performed siRNA-mediated functional knocking-down studies in the mouse pancreatic beta cell line, βTC3 [50]. Control (lacZ) and Rfx6-specific siRNAs were transfected into βTC3 cells. Subsequently, total RNAs were isolated from the transfected βTC3 cells and analyzed by semi-quantitative RT-PCR. Semi-quantitative RT-PCR analysis showed that transfection of Rfx6 siRNA, not LacZ siRNA, caused a significant decrease of Rfx6 mRNA level in βTC3 cells (Fig. 3.4A). This suggests that the synthetic Rfx6 siRNA specifically targeted endogenous Rfx6 mRNA for degradation. To assess whether knocking-down of RFX6 affects pancreatic gene expression, we analyzed the mRNA expression of 12 islet-specific transcription factors, including Beta2, Hes1, Isl1, IA1, MafA, Ngn3, Pax6, Nkx2.1, Nkx6.1, Pax4, Pax6 and Pdx1. No significant alteration of Beta2, Hes1, Isl1, IA1, MafA, Ngn3, Nkx2.1, Pax4, Pax6 and Pdx1 expression was detected between LacZ and Rfx6 siRNA transfected cells (data not shown). However, *Nkx6.1* expression was down-regulated nearly 2 folds in Rfx6-siRNA transfected βTC3 cells (Fig. 3.4B). Based on these observations, we speculated that RFX6 may be directly or indirectly involved in the transcriptional regulation of Nkx6.1 in pancreatic β cells. To test this hypothesis, we generated a luciferase reporter construct containing 1.3-kb of the 5' flanking region of the mouse Nkx6.1 gene. This proximal promoter region of Nkx6.1 was previously shown to contain a β-cell specific enhancer [51]. As a control, we also generated a luciferase reporter construct containing 1.8-kb of the 5' flanking region of the mouse Rfx6 gene. We then co-transfected the luciferase reporter constructs with an

expression plasmid encoding either GFP or RFX6 into HEK293 (non-β) and Min6 (β) cells. Luciferase analysis showed that, in both β and non¬β cell lines, ectopic expression of RFX6 markedly activates the Nkx6.1, but not the Rfx6 proximal promoter (Fig. 3.4C-D). These results, together with the siRNA knocking-down data, strongly indicate that RFX6 functions as a transcriptional activator for the endocrine lineage specific gene Nkx6.1 in matured pancreatic β cells.

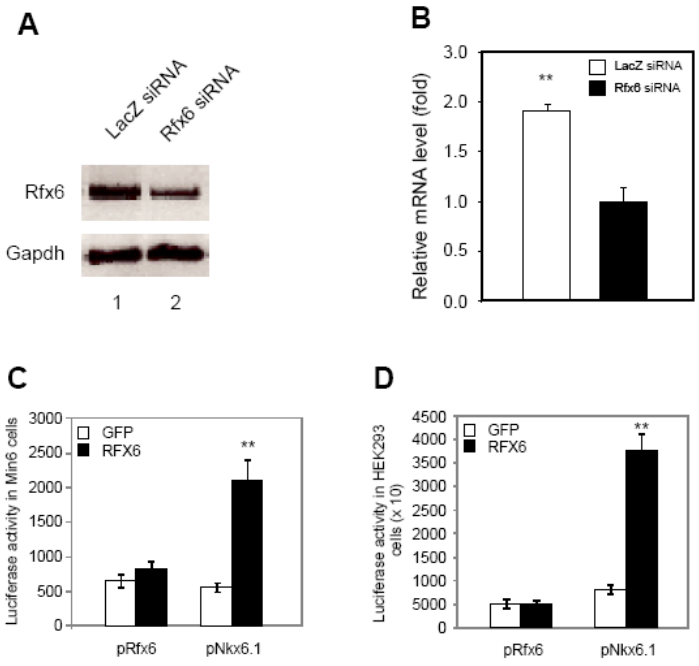

Figure 3.4 RFX6 functions as a transcriptional regulator for the endocrine lineage-specific gene Nkx6.1. (A) Semi-quantitative RT-PCR analysis of Rfx6 mRNA expression in βTC3 cells transfected with lacZ and Rfx6 specific siRNAs. Note that Rfx6 mRNA level was significantly decreased in Rfx6-specific siRNA transfected βTC3 cells, suggesting Rfx6 siRNA specifically targeted Rfx6 mRNA for degradation. (B) Quantitative RT-PCR analysis of Nkx6.1 mRNA expression in LacZ and Rfx6 specific siRNA-treated βTC3 cells. The value of Nkx6.1 mRNA expression in Rfx6 siRNA-treated βTC3 was set to 1 and used to calculate relative expression

level in LacZ siRNA-treated βTC3 cells. N = 3 independent transfections, *, $p < 0.05$, **, $p < 0.01$ LacZ versus Rfx6 siRNA-treated βTC3 cells. (C-D) Quantitative analyses of Rfx6 and Nkx6.1 promoter (pRfx6 and pNkx6.1) activities in Min6 (C) and HEK293 (D) cells transiently expressing GFP or RFX6. Min6 and HEK293 cells were transiently co-transfected with expression plasmids encoding either GFP or RFX6 with luciferase reporter constructs containing either the mouse Rfx6 or Nkx6.1 gene proximal promoter. An expression plasmid encoding β¬galactosidase (βgal) was included in all transfection and was used as internal control to normalize transfection efficiency among different groups. Luciferase assay was performed 24 hours after transfection. N = 3 independent transfections, *, $p < 0.05$, ** , $p < 0.01$ GFP versus RFX6-expressing cells.

Rfx6 is required for islet formation during zebrafish embryonic development

To assess the role of Rfx6 in control of pancreatic development, we made use of the zebrafish model. RNA in situ hybridization was used to analyze the spatial-temporal expression of the zebrafish Rfx6 gene. Rfx6 transcripts were detected at the pharyngula stage (36 hours post fertilization, hpf) in the pancreatic epithelium (Fig. 3.5A, arrow). To assess the role of Rfx6 in regulating zebrafish pancreatic development, LacZ- (control) and Rfx6-specific morpholino antisense oligonucleotides (MO) were injected into one-cell stage zebrafish embryos. Injection of Rfx6-specific MO caused a significant decrease of Rfx6 mRNA in zebrafish embryos as compared to LacZ MO injected embryos (Fig. 3.5B), suggesting that the Rfx6-specific MO specifically targeted Rfx6 mRNA for degradation. MO injected embryos were allowed to develop for 36 hours and then subjected to RNA in situ hybridization using a digoxygenin-labeled insulin antisense riboprobe. At 36 hpf, Rfx6 MO-injected embryos showed either a decreased insulin expression (Fig. 3.5D and G)

or a disorganized islet (Fig. 3.5G and 3.5H), compared to control MO injected embryos (Fig. 3.5C and F). The observed pancreatic phenotypes correlated positively with the dose of the MO and, importantly, were rescued by co-injection of the morpholino oligonucleotides with *Rfx6* synthetic mRNA (Fig. 3.5I). We quantified the expression of insulin mRNA in control and Rfx6-spefic MO injected embryos using real-time RT-PCR. Insulin expression was significantly decreased by the injection of *Rfx6* MO (Fig. 3.5J). Together, these data suggest that RFX6 is critical for beta cell differentiation and islet formation during zebrafish pancreatic development.

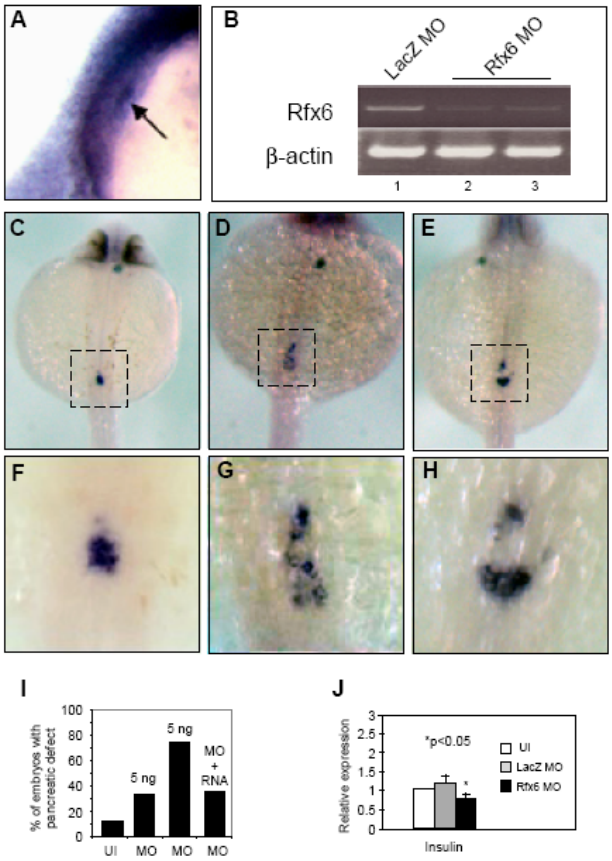

Figure 3.5 Knocking-down of RFX6 function in zebrafish impairs β-cell development and insulin expression. (A) RNA in situ hybridization of zebrafish embryos at the pharyngula stage using an antisense zebrafish Rfx6 probe. Rfx6 mRNA was detected in the pancreatic primordial (arrow). (B) Semi-quantitative RT-PCR

analysis of LacZ (lane 1) and Rfx6-specific (lane 2 and 3) morpholino (MO) oligos injected embryos. Injection of Rfx6-specific morpholino oligonucleotides results in decreased levels of Rfx6 mRNA in zebrafish embryos. (C-E) RNA in situ analysis of insulin mRNA expression in LacZ (C) and Rfx6 (D-E) MO injected zebrafish embryos. LacZ MO injected embryos had reduced insulin expression (D) or impaired islet morphology (E). (F-H) Magnified views of the boxed areas in C-E, respectively. (I) Quantification of zebrafish embryos with pancreatic defects. (J) Quantitative RT-PCR analysis of insulin mRNA expression in uninjected (UI), LacZ and Rfx6 MO injected embryos. N = 3 independent injections, *, $p < 0.05$ Rfx6 versus LacZ MO injected embryos.

3.4 Discussion

The endocrine and exocrine lineages of the pancreas derive from a transient pool of pancreatic progenitors during embryonic development. The genetic determinants underlying endocrine pancreas formation, function and survival are not completely understood. Through differential gene expression screening, we have identified a novel mouse gene, *Rfx6*. We show here that *Rfx6* encodes a nuclear protein with a winged-helix DNA-binding domain. *Rfx6* is exclusively expressed in the islets of Langerhans of the pancreas in adult mice. Knocking-down of *Rfx6* function in pancreatic β cells using siRNA causes significant down-regulation of *Nkx6.1* mRNA expression. Consistent with this, ectopic expression of *Rfx6* in both non-β and β cells activates the *Nkx6.1* proximal promoter. *Rfx6* is expressed in the developing zebrafish pancreas at the pharyngula stage. Morpholino antisense oligo-mediated knocking-down of Rfx6 function attenuates the differentiation of insulin-expressing cells and impairs islet morphology. Taken together, these results strongly suggest that RFX6 functions as a transcriptional activator for the islet-specific gene

Nkx6.1 in adult β cells. Our data also indicate that RFX6 may be an important regulator of islet cell formation during vertebrate embryonic development.

Members of the RFX family share a highly conserved 76-amino acid winged helix DNA binding domain [45]. This domain binds to the X-box motifs in the regulatory region of target genes [38, 52]. We show by sequence alignment analysis that the deduced RFX6 peptide shows the highest sequence homology (36.6%) to RFX4. This suggests that Rfx6 may be derived from Rfx4 through a gene-duplication event during evolution. Sequence analysis also shows that, unlike other RFX proteins, RFX6 contains 7 dileucine motifs (Fig. 3.1). Of these 7 dileucine motifs, three are the "DxxxLL" type that has previously been implicated in interactions with the heterotetramer assembly protein 2 (AP-2) adaptor protein complex of the clathrin-coated vesicles (CCV) [53]. "DxxxLL" type dileucine motifs have been found in many membrane receptors and their ligands and is used by the cellular sorting machinery as an internalization signal [54, 55]. We have demonstrated, through ectopic expression of RFX6 in Hela cells, that RFX6 is predominantly localized in the nucleus of mammalian cells. More importantly, we show that siRNA-mediated knocking-down of RFX6 causes significant down-regulation of Nkx6.1 gene expression. Furthermore, ectopic expression of RFX6 activates the Nkx6.1 gene proximal promoter. Together, these data suggest that RFX6, as other members of the RFX protein family, functions as a transcription factor. It is important to point out, however, that it is possible that a small amount of RFX6 may be localized in the cytoplasm and thus may play a role in intracellular protein sorting. Further molecular and biochemical studies are needed to define the role of the multiple dileucine motifs in RFX6.

Rfx6 mRNA is exclusively expressed in the islets of Langerhans of the pancreas. Through siRNA-mediated functional knocking-down and promoter reporter assays in βTC3 cells, we demonstrate convincingly, that RFX6 positively regulates the

transcription of the mouse Nkx6.1 gene. Previous studies have shown that the homeobox gene Nkx6.1 plays an essential role in regulating the differentiation of the endocrine lineage of the pancreas during mouse embryonic development. Targeted disruption of Nkx6.1 in mice leads to loss of β-cell precursors and blocks β-cell differentiation during the secondary transition [29]. More recently, it has been shown that over-expression of Nkx6.1 in both human and rat islets stimulate β-cell replication with retention or enhancement of β-cell function, suggesting that Nkx6.1 is critically required for β-cell growth in adult mice [56]. Given the clear involvement of Nkx6.1 in control of β-cell replication, we speculate that RFX6 may play an important role in regulating β-cell mass in adult mice. Proof of this hypothesis will require genetic and molecular studies in mice.

In conclusion, we have identified a novel islet-specific factor, RFX6, in mice. Here, we provide several lines of experimental evidence suggesting that RFX6 functions as a transcriptional regulator for the homeobox gene Nkx6.1. While this manuscript was in review, two papers describing the expression and function of RFX6 during vertebrate pancreas development were published [46, 47]. Our findings, together with the recently reported functional data on RFX6, have important implications in understanding the molecular mechanisms regulating β-cell development and growth in vertebrates.

3.5 Materials and Methods

Ethics Statement

All mouse work was performed under approved Cornell University Animal Protocol # 2006-0087. All zebrafish work was performed under approved Medical College of Wisconsin Animal Protocol # 312-06-2.

DNA Constructs and luciferase assay

Two promoter constructs tethered to a luciferase reporter gene were used: pRfx6-Luc and pNkx6.1-Luc. To generate pRfx-Luc, the 5' flanking region of the mouse Rfx6 gene (¬1837 to +1) was amplified by PCR using the following primers: 5'¬GCTCTTACGCGTGCCCCTGTAGAGTAGGTTATC-3'(forward) and 5'-GATCGCAGATCTCCTTCCCACCGCCCGGAT-3' (reverse). The PCR-amplified Rfx6 promoter fragment was cut with Mlu I and Bgl II and the resulting fragment was subcloned between the Mlu I and Bgl II sites of the pGL3-Basic luciferase vector (Promega, Madison, WI). To generate pNkx6.1-Luc, the 5' flanking region of the mouse Nkx6.1 gene (-1317 to +1) was amplified by PCR using the following primers: 5'- ACGCGTGCTGGCTCTAGACTGGAA -3' (forward) and 5'¬GGATCCCCCACAGCCAGACCCGAA-3' (reverse). The PCR-amplified Nkx6.1 promoter fragment was first cloned into pGEM-T vector (Promega, Madison, WI). The Nkx6.1 promoter fragment was then released from pGEM-T vector by Mlu I and Bam HI double digestion and subcloned between the Mlu I and Bgl II sites of pGL3-Basic luciferase vector. The RFX6-Flag expression construct, pRfx6-Flag, was generated through the following steps: First, pDNR-CMV-Flag vector was modified from pDNR¬CMV vector (Clontech, Mountain View, CA) by creating an adaptor using two synthetic oligonucleotides: 5'-AGCTTGACTACAAAGACGAT GACGACAAGTGAG-3' (forward) and 5'-CGCGCTCACTTGTCGTCATCGTCTTT GTAGTCA-3' (reverse). Subsequently, the open reading frame region of the mouse Rfx6 cDNA was amplified by PCR using the following primers: 5'-TTGAATTCCGCCACCATGGCTAAG GTCCGGGAA -3' (forward) and 5'-TTCTCGAGAGTGTCTCCAGCTGCTGT -3' (reverse). The PCR products were cut with Eco RI and Xho I and the resulting fragments were subcloned between the Eco RI and Xho I sites of pDNR-CMV-cFLAG vector. All plasmid constructs were

confirmed by restriction enzyme digestion and sequencing. For luciferase assay, HEK293 or Min6 cells were transfected using polyethylenimine (PEI) (Sigma) with 3.5 μg of DNA mix containing 0.5, 1 and 2 μg of pCMV-βgal (transfection control), different luciferease reporter construct and pRfx6¬Flag or pEGFP-N2, respectively.

Islet Isolation

Mouse islet isolation was performed using collagenase digestion and Ficoll gradient centrifugation as previously described (57). Briefly, after anesthetizing each mouse, the pancreas was perfused with 1 x Hank' Balanced Salt Solution (HBSS) (pH7.4) containing 2.0 mg/ml of type V collagenase (Sigma). The inflated pancreas was dissected and incubated at 37^{o}C for 20 minutes. The collagenase-digested pancreas was vigorously shaken for 5 seconds to release islets from acinar tissues, washed three times with 5-7 mls of ice-cold 1 x HBSS buffer and re-suspended in 2 mls of 28% Ficoll (VWR). The islet and acinar mixture was loaded onto the top of a gradient Ficoll solution as previously described and centrifuged at 2250 rpm for 7 minutes at 4oC. Islets were collected and washed three times with cold 1 x HBSS and processed for further analysis.

RNA isolation and quantitative RT-PCR

RNA isolation was performed using the TRIZOL RNA Isolation Kit (Invitrogen, Carlsbad, CA). For quantitative RT-PCR analysis, the tissue-specific RNAs were treated with DNase I for 10 minutes and purified using the RNAqueous-Micro Kit (Ambion, Austin, TX). First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using Power SYBR Green PCR Master Mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Briefly, 25 ng of total cDNA was mixed with Power SYBR Green PCR Master solution (Applied Biosystems) in a 25 μl reaction. Quantification and normalization of mRNA expression was performed

essentially as described (58). Expression difference between compared groups was statistically analyzed by performing Student's t-tests using the normalized mean value

Transfection and immunohistological analysis of Hela cells

Hela cell transfection was done using polyethylenimine (PEI) (Sigma). Hela cells were seeded at a density of 0.5 x 106 cells per well in 6-well dishes with cover slips. After an overnight rest, Hela cells were rinsed twice with PBS, once with Opti-MEM reduced serum medium (Invitrogen) and incubated for 12-16 hours with 1 ml of Opti-MEM reduced serum medium containing 3 µg of expression plasmid encoding RFX6-FLAG and 2 µl of PEI. Transfected Hela cells were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for over 2 hours. Immunostaining was performed essentially as described (59, 60). Briefly, Hela cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min, washed 3 times in 0.1% Triton X-100 in PBS and 3 times in PBS. The permeabilized sections were preincubated with 5% normal donkey serum and 1% BSA in PBS at room temperature for at least 1 hour, followed by incubation in the same solution with a mouse monoclonal anti-FLAG antibody (Sigma, 1:1000) at 4°C overnight. The antibody-bound sections were washed three times in 0.1% Triton X-100 in PBS, 3 times in PBS alone and then incubated with Cy3-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, 1:500) for 1–3 hrs at room temperature. After extensive washing, fluorescence images were acquired using an Axiovert 40 microscope (Zeiss) equipped with an AxioCam camera.

Culture of βTC3 cells and siRNA transfection

βTC3 cells were grown under conditions described previously (50). The mouse Rfx6 coding region corresponding from +45 bp to +501 bp was amplified using the following primers: forward: TCTGCACCCATTCTCAACTG and reverse: CTCTTGTGTGTGTGTGGATGG. Control (lacZ) and Rfx6-specific siRNA were

generated using the BLOCK-iTTM Complete Dicer RNAi Kit (Invitrogen) according to the manufacturer's recommendations. βTC3 cells were grown to 80% confluence and transfected with 100 ng of either lacZ- or Rfx6-specific siRNA per plate using LipofectamineTM 2000.

Zebrafish studies

All zebrafish work was performed under approved Medical College of Wisconsin Animal Protocol # 312-06-2. Antisense and sense digoxygenin-labeled riboprobes were synthesized using Sp6 and T7 RNA polymerase, respectively using template plasmid linearized with SacII and SalI, respectively. In situ hybridization using antisense Rfx6 digoxygenin-labeled RNA probe was performed as described previously (61). The Rfx6¬specific (5'-AATAAAAACGCCTCTTACCTTTCCG) and standard control (5'¬CCTCTTACCTCAGTTACAATTTATA) morpholino oligos were designed by GeneTools Inc. Morpholino (5-10 ng) and RNA (100-300 pg) injections were performed essentially as described (61). Primers used for detecting Rfx6 MO efficacy include: F: GCAAGCATCTCCTCTGAAGAGA, and R: CACTGCTCTCCTTGATTCCAATA. Zebrafish Actin primers were described previously (61). Capped mouse sense RNA was generated with SP6 polymerase from a NotI linearized pCS2.1 template containing Rfx6 gene using the Ambion mMESSAGE kit. For rescue experiments, 10 ng of MO and 200 pg of capped sense mouse Rfx6 RNA was co-injected per embryo. Insulin mRNA expression was analyzed by quantitative RT-PCR using the following primers: insulin-F: 5'-AGTGTAAGCACTAACCCAGGCACA; insulin-R: 5'¬TGCAAAGTCAGCCACC TCAGTTTC.

Statistical analysis

Differences between groups were evaluated by performing two-tailed Student's t-test and $p < 0.05$ is considered significant.

3.6 Acknowledgements

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APPENDIX

SPECIFIC CONTRIBUTIONS TO EACH CHAPTER

In using paper option, Shuai Li wrote chapter 1 of the thesis. Chapter 2 and chapter 3 are individual manuscripts written by Dr. Qiaoming Long.

In chapter 2, Robert Munroe from Dr. John Schimenti's lab conducted the blastocyst injection. Adam Francisco from Dr. Qiaoming Long's lab maintained the mice line. Dr. Qiaoming Long conducted all the statistic analysis for figure 2.2-2.7. Shuai Li conducted the immunohistochemistry studies for all the panels in figure 2.1-2.6 and 2.8. Shuai Li also conducted the genotyping analysis presented in figure 2.1.

In chapter 3, Dr. Qiaoming Long conducted the bioinfomatic studies for figure 3.1. Zebrafish studies were conducted in collaboration with Dr. Ramani Ramchandran's lab at University of Wisconsin, Madison. Shuai Li analyzed the expression profile of *Rfx6* for figure 3.2 and figure 3.3, the expression of *Rfx6* and *Nkx6.1* for figure 3.4. Finally, Shuai Li conducted the luciferase reporter assay for figure 3.4.